DETERMINING THE MECHANISM OF ACTION OF A NOVEL CANCER THERAPEUTIC

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Mona Ahmad

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DETERMINING THE MECHANISM OF ACTION OF A NOVEL CANCER THERAPEUTIC

Approved by:

Dr. Ravi Bellamkonda, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Jung Choi
School of Biology
Georgia Institute of Technology

Dr. Joseph Montoya
School of Biology
Georgia Institute of Technology

Date Approved: [Date Approved by Committee]

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SUMMARY

Glioblastoma is an extremely invasive form of brain cancer, causing it to be very deadly even after administering therapy. The cells of glioblastoma invade the tissue surrounding the brain tumor, so that the cancer persists even when the tumor is removed. To address this problem, Imipramine Blue, a novel cancer therapeutic has been developed. The drug has been successful in halting invasion of cancer cells *in vitro* and *in vivo*, but its mechanism of action is unclear. From examining the normal mechanism of cell invasion, it seems reasonable to expect that Imipramine Blue binds to actin or one of its upstream regulators. To examine the mechanism of action, actin polymerization assays, immunocytochemistry, nuclear localization, and a magnetic bead binding assay were performed. The data from the actin polymerization assays shows that Imipramine Blue does not affect actin polymerization outside of the cell. The immunocytochemistry data confirms that Imipramine Blue does not affect the actin structures or upstream regulators in healthy cells, but changes the activity of these proteins in cancer cells. Imipramine Blue has been shown to localize to the nucleus in nuclear localization assays. Finally, the magnetic bead binding assay identifies a splice variant of Nox4, an upstream regulator, as the binding protein to the drug. Identifying the mechanism of action of Imipramine Blue ensures that researchers and doctors can confidently apply the drug to other cancers, knowing that it will not interact with healthy cells.

CHAPTER 1

INTRODUCTION

1.1 Background

Glioblastoma is an extremely invasive and deadly form of brain cancer. The five year survival rate of a patient diagnosed with this cancer is only 35%. The cells of glioblastoma can reproduce quickly and tend to migrate along the white matter tracts of the brain, which causes the tumor as a whole to be invasive throughout. The popular treatment for glioblastoma is resection; this is when a surgeon removes the tumor from the brain tissue. However, this surgery adds only a year at most to the patient's lifespan. The cells that have migrated away from the main body of the tumor eventually reproduce and grow into a new tumor, further damaging the brain. Radiation is also ineffective as a treatment; if it is not aimed directly at the tumor, the surrounding brain tissue will be damaged, making radiation of the migrating cells impossible. Therefore, it makes sense that halting invasion of the tumor is a priority if doctors would like resection and radiation to be effective.

The Bellamkonda lab has identified a novel compound that is anti-invasive to cancer called Imipramine Blue, provided by the Arbiser lab at Emory University. During *in vitro* experiments, such as invasion assays, the compound was shown to halt invasion of the cancer cells after only 3 hours; imaging also proved that the cells lost their invasive structures during this time (Figure 1).

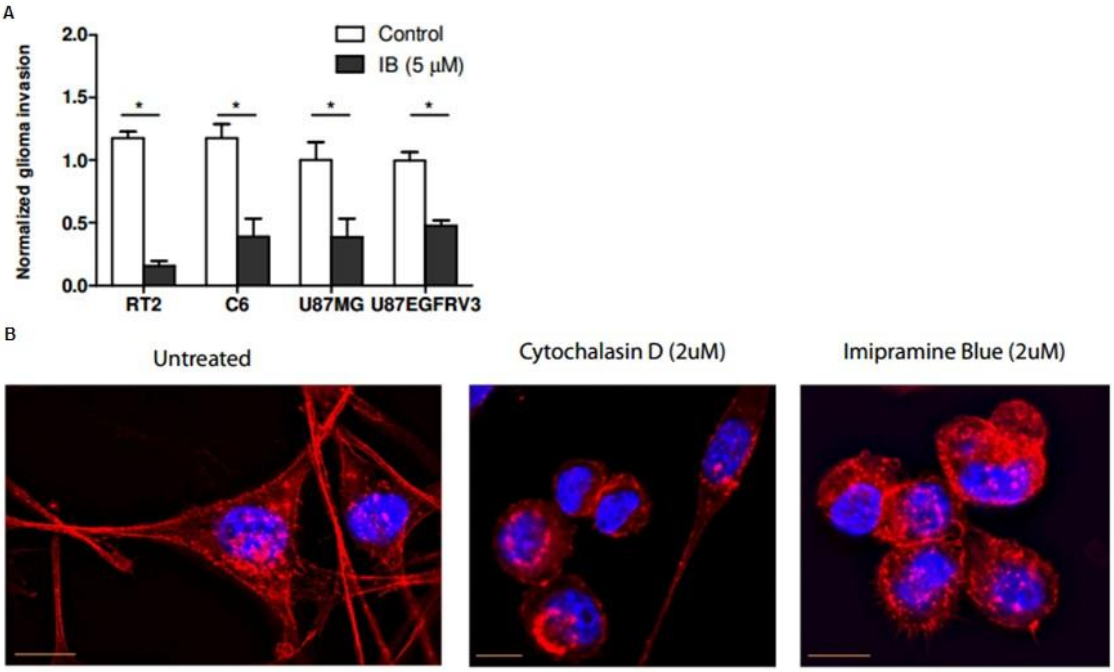

Figure 1: IB is anti-invasive *in vitro*. (A) Invasion assays were performed with RT2, C6, U87MG, and U87EGFRV3 cells. IB was found to decrease invasion by the greatest amount in RT2 cells, a rat glioblastoma cell line. (B) RT2 cells were treated with Imipramine Blue (drug), treated with cytochalasin D (actin inhibitor), or untreated. After 3 hours, cells were fixed with 5% paraformaldehyde and stained for the cytoskeleton (red) and the nucleus (blue). IB was shown to have similar effects to cytochalasin D, thus halting invasion. Adapted from Munson 2012.

Imipramine Blue also shows promise as a cancer therapy through its *in vivo* studies. Rats that were inoculated with a brain tumor and then treated with Imipramine Blue displayed tumors that showed no migration or spreading; normal brain tumors will be very spread out and the body of the tumor will be difficult to find (Figure 2).

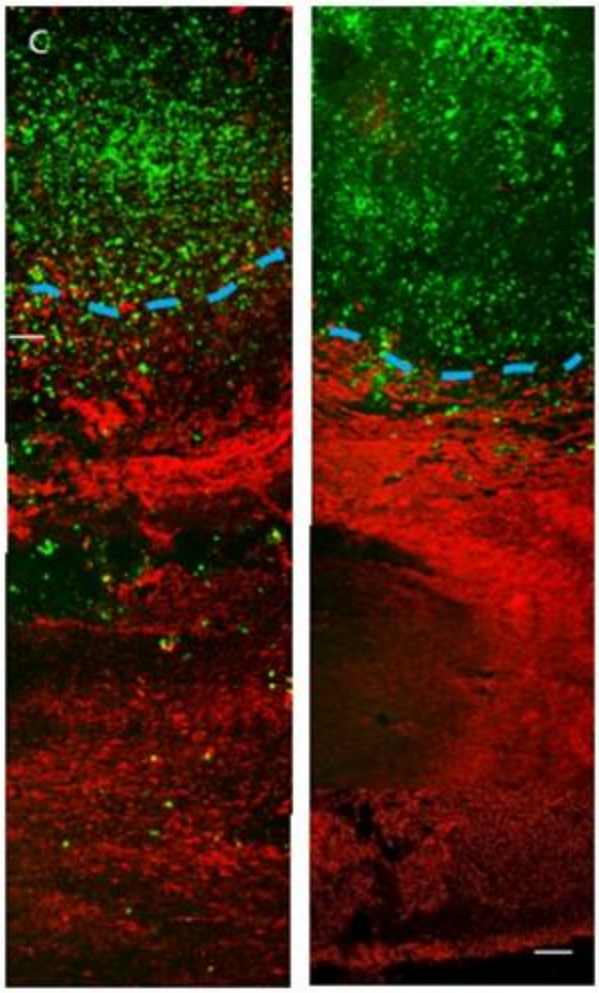

Figure 2: IB is anti-invasive *in vivo.* 3RT2RT1 glioma cells were inoculated into rats' brains. The brain on the left has not been treated and the brain on the right has been treated with IB. Brain slices were stained for the tumor (green) and healthy tissue (red) and imaged using fluorescence microscopy. The brain on the left shows cell spreading past the tumor border, whereas the IB treated brain is more clustered and stays within the confines of the border. Adapted from Munson 2012.

When the tumors underwent additional chemotherapy after Imipramine Blue treatment, all evidence of the glioblastoma disappeared in the brain (Figure 3).

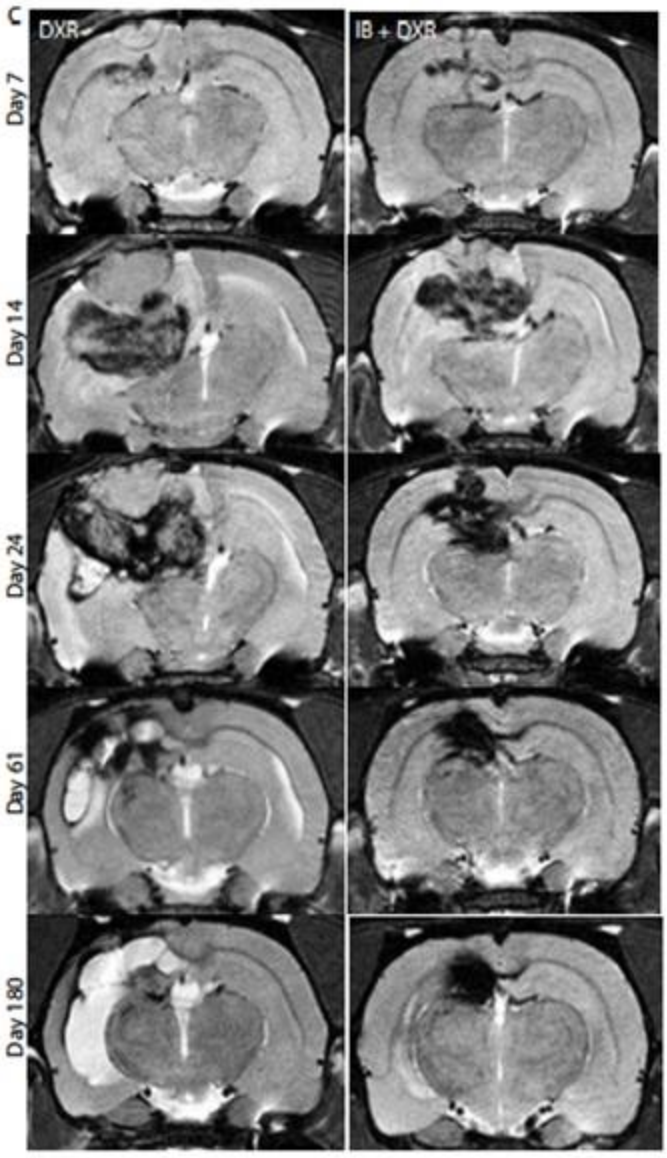

Figure 3: Co-treatment of IB and Doxorubicin *in vivo* causes total remission of glioblastoma. Rats were given 3RT1RT2 tumors and underwent two separate treatments: one group had doxorubicin alone (right) and the other had a co-treatment of doxorubicin and IB (left). The DXR rats showed tumor regrowth after 180 days; the IB rats showed complete remission. Adapted from Munson 2012.

These results are promising for the future of Imipramine Blue and highlight its potential as a cancer therapeutic.

While it is clear that Imipramine Blue can stop glioblastoma invasion, its mechanism of action is still unknown. Understanding how Imipramine Blue works is

important to show that it is truly not harmful to healthy cells in any way. This way, the drug can be tested in bigger animals with more confidence. Additionally, by understanding the mechanism of action of Imipramine Blue, researchers can know which others cancers the drug would potentially work on. Having one drug to treat multiple cancers would be advantageous because it is cost-effective.

Using the experiments that the Bellamkonda lab has already done, a starting point for this project would be to examine the cells at the protein level. The cancer cells become noninvasive because their cytoskeletal structures changed through actin rearrangement. Therefore, it is necessary to study the proteins that contribute to migration, starting with actin. Other protein pathways that lead to actin rearrangement, such as the Nox4 pathway, are also important for study. Lastly, the proteins that lead to matrix degradation of the surrounding brain tissue are worth studying as well if intercellular proteins do not yield any results.

1.2 Specific Aims

1) Localize Imipramine Blue in RT2 cells *in vitro*

2) Differentiate levels of select proteins in Imipramine Blue treated cells at various times

3) Characterize specific Imipramine Blue binding protein

1.3 Literature Review

1.3.1 Introduction

To fully understand how Imipramine Blue halts invasion, the literature discussing the invasive properties of glioblastoma must be examined. The invasive mechanisms of glioblastoma are well-established. By fully understanding the normal mechanism of

migration, it is possible to understand how Imipramine Blue disrupts it. In this review, I will summarize the literature describing the most common mechanisms of glioblastoma invasion, including chemical signals from the microenvironment and signals from within the cell itself. The research in this literature review will identify which proteins should be given priority in this study, based on how central they are to glioblastoma migration.

1.3.2 Cytoskeleton Rearrangement

A cell cannot migrate without actively rearranging its cytoskeleton. Non-invasive cells are typically spread out in every direction. Conversely, an invasive cell has protrusions coming from the cell body that help it to migrate. Yamaguchi and Condeelis (2007) thoroughly describe the importance of actin to cancer cell movement in their research. They show in their work that disrupting normal actin function in glioblastoma effectively takes away its ability to move. The results found from Imipramine Blue treatment mimic the results of this study: cells become rounded and lose all motility. When the cell migrates, the actin cytoskeleton must rearrange to create an invasive structure; these structures are identified by Yamaguchi and Condeelis as filopodia, lamellipodia, and specifically for cancers, invadopodia. The invadopodia can be seen as a protrusion from the cell, projecting into the extracellular matrix. The researchers also identify profilin and scinderin as active regulators of actin polymerization; these can stop or start the process of actin rearrangement[13]. Hall and Nobes (2000) explain that actin is regulated upstream by RhoGTPases, Src kinase, and NFκB. Disrupting the function of any of these upstream regulators causes similar results as those in Yamaguchi's study.

It is clear from this research that actin is the most likely candidate for Imipramine Blue binding and disruption and a starting point for this study. Interfering with actin shows similar morphological results as the results obtained from Imipramine Blue treatment. The study will begin by examining actin and its regulators, profilin and scinderin. Following that, the upstream regulators identified by Hall and Nobes will be considered. If none of these prove to be the protein of interest, the study must broaden to look at proteins even farther upstream.

1.3.3 NADPH Oxidase Signalling

There is another class of proteins which causes the cell to migrate in conjunction with the actin family, called NADPH Oxidases, or the Nox family of proteins. Chen, Craige, and Keaney (2009) explain in their study of the Nox family that Nox proteins cause reactive oxygen species (ROS) to generate in the cell[1]. This is significant because ROS activate many signaling pathways relating to cell survival, namely cell migration. Because of the effects of ROS, cancer cells exploit Nox proteins to their advantage, improving their survival. The researchers categorize six types of Nox proteins: Nox1, Nox2, Nox3, Nox4, Nox5, and Duox1/Duox2, all which produce ROS in cancer cells. Chen and his collaborators found that the categories of Nox proteins are located in different membranes within the cell and that the location of the Nox protein determines its downstream effects. The membranes the Nox family can be on include the endoplasmic reticulum, the nucleus, the mitochondria, the outer cell membrane, or on individual endosomes[1]. The Nox proteins can affect actin rearrangement from all of these areas of the cell. One step in the research is to identify where Imipramine Blue is

localizing within the cancer cell; this way, if Nox is found to be the binding protein, it will be possible to identify its category. Recently, Goyal (2009) has shown that Nox can be found in cells as a "splice variant." This means that the protein has been cleaved but still retains its function. Goyal's research shows that these splice variants also have separate locations in the cell, further complicating the effort to narrow down the possible proteins in this study. For example, Nox4, identified by Munson (2011) as an important protein to study for glioblastoma, has five different splice variants which can be found in the endoplasmic reticulum, the nucleus, and the cytoplasm[6].

Nox proteins are worth a lot of study when trying to understand brain cancer because they are so central to cancer migration. The ROS they create activate many proteins that are crucial to beginning the invasion process. In a Nox study by Shono (2008), he shows that protein kinase C, protein tyrosine phosphatases, and mitogen-activated protein kinase are all activated by ROS, which causes cells to become malignant and cancerous[12]. Nox4 has specifically been implicated by Li (2009) and Munson (2011) in causing many of the changes seen in glioblastoma; it is overexpressed in every stage of glioblastoma invasion. When Nox4 was knocked out in Li's research, the glioblastoma became significantly less invasive and responded well to cancer therapies. In the same study, Nox4 was needed for creation of invadopodia[9]. It is clear that special attention should be given to Nox4 in the study of glioblastoma invasion if actin is not identified as the central binding protein.

1.3.4 Extracellular Matrix Detachment and Degredation

Gimona (2008) has examined the extracellular environment in his study of cell movement. He found that the first step to cell invasion through the matrix is detachment from the extracellular matrix (ECM) and from other cells. Cells stay adherent to the ECM through focal adhesions, which interact with many other proteins to maintain adherence[5]. Demuth and Berens (2004) have identified the proteins as integrins, Src kinase, and focal adhesion kinase (FAK). These are regulated by RhoGTPase; Src kinase is also regulated by interacting with FAK[2]. They discovered that proteins work to maintain cell binding and change the internal structure of the cell by recruiting cytoskeletal proteins. Research by Friedl and Wolf (2009) examining cell migration has shown that FAK is overexpressed in many cancer types; the degree of overexpression correlates to the degree of malignancy. Src is similar in this property: overexpression correlates to malignancy. They realized that that turning these proteins off in the cell can halt invasion[4]. It is unclear from previous research whether Imipramine Blue stops the cell from binding to the extracellular matrix, or if it just does not allow the cell to rearrange internally. Therefore all of the proteins identified in the research characterizing cell movement through the matrix should be under scrutiny.

For cells to continue invading through the extracellular matrix, it is necessary for them to degrade the matrix itself. A very broad study by Pullen and Fillmore has shown that this is most commonly done using matrix mettaloproteinases (MMPs). MMPs degrade membrane proteins such as collagen and gelatin, which hold the matrix together. Two types, MMP-2 and MMP-9, are extremely overexpressed in glioblastoma. In the study, an experiment demonstrated that MMP activity can be induced by a variety of

signals, including normal cells of the brain and that inhibiting MMP activity successfully halts invasion.

Since Imipramine Blue is able to halt invasion *in vitro*, where there is no collagen, gelatin, or laminin to break down, these are most likely not the proteins of interest for this study. However, preliminary experiments using laminin coated plates have shown similar results to experiments preformed without laminin. Therefore, MMPs are important proteins to keep in mind if the study does not yield any other results.

CHAPTER 2

IMIPRAMINE BLUE LOCALIZATION

2.1 Introduction

To first understand how Imipramine Blue is halting invasion of cancer cells, we must first know where Imipramine Blue localizes in treated cancer cells. By attaching a fluorescent marker to Desipramine Blue, an analog of Imipramine Blue which has a conjugation site, we will be able to track the drug in the cell by imaging at different time points. Once we see where the drug acts in the cell, we can narrow down our host of proteins according to cell region (nuclear, mitochondrial, cytosolic, etc.)

2.2 Methods

2.2.1 Desipramine Blue Conjugation and Localization

Desipramine Blue (DB) was conjugated to amine reactive FITC (Invitrogen) using the protocol provided. Conjugated DB was then encapsulated in liposomes in preparation for delivery. RT2 cells were seeded at 100,000 cells/ml in four 8 chamber slides. Each slide will undergo a different drug treatment (liposomal conjugated DB, free conjugated DB, free IB, untreated). Cells were then fixed with 4% paraformaldehyde at the following timepoints: 5 minutes, 15 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 48 hours. Cells were stained with rhodamine phalloidin (Invitrogen) and DAPI before imaging.

2.3 Results

2.3.1 Imipramine Blue localizes to the nucleus of RT2 cells

Following treatment with DB, cells were imaged for FITC, DAPI (showing the nucleus), and rhodamine phalloidin (showing actin) at each timepoint mentioned. The

treatments imaged were the DB-FITC conjugate, free DB, free FITC (not shown) and a control. After imaging, we observed FITC overlapping with DAPI in the DB-FITC treatment; in other words, FITC was brightest in the nucleus (Figure 4). Additionally, the experiment showed that treatment with DB causes RT2 cells to lose their invasive actin structures (Figure 4).

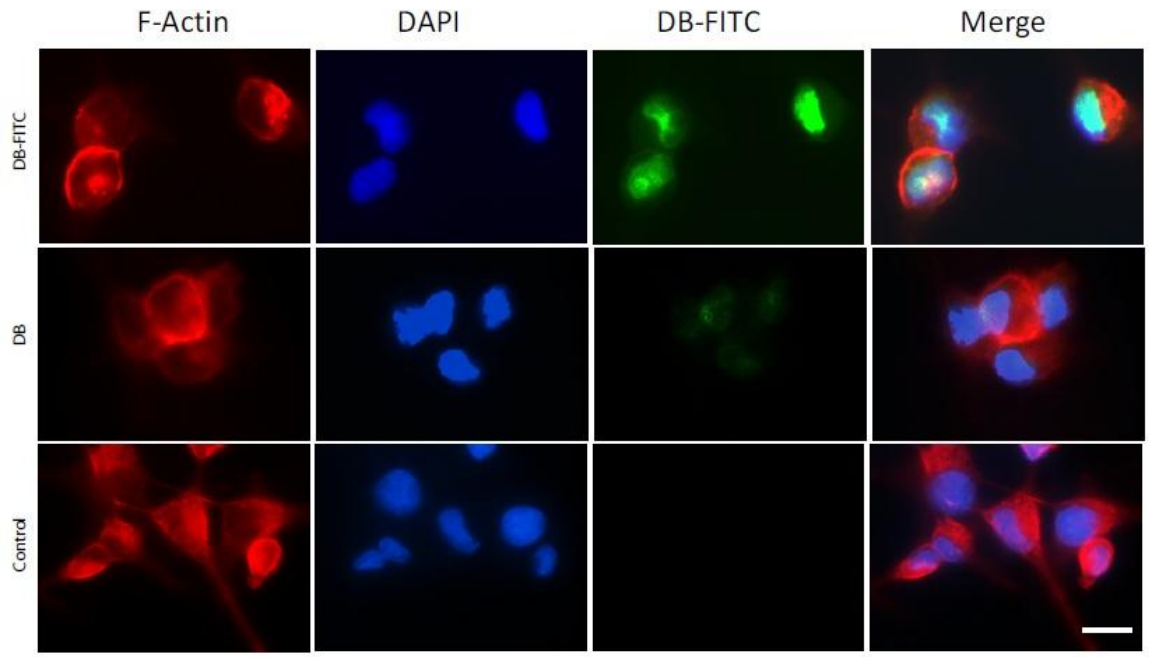

Figure 4: DB-FITC localizes to the nucleus in RT2 cells. DB was conjugated to a FITC tag and administered to RT2 cells *in vitro*. Cells were then stained for F-Actin (red), nucleus (blue), and DB-FITC (green). Results showed that DB-FITC localizes to the nucleus; additionally, DB alone affects RT2 cells in a similar manner to IB.

2.4 Discussion

The results of the experiment showed that Desipramine Blue localizes to the nucleus. This is evidenced by the images, which have a bright FITC overlap with DAPI, the nuclear stain (Figure 4). We can assume that DB has the same activity as IB *in vitro* because the experiment also showed that it causes the same rounding in cells as IB treatment does. Therefore, IB localizes to the nucleus in RT2 cells. Proteins in the nucleus are usually regulators of transcription; because of their activity, the results of this

experiment show that IB most likely binds to an upstream protein in the invasion pathway. Knowing where IB acts in the pathway can help us to choose specific proteins for future study.

CHAPTER 3

IN VITRO PROTEIN LEVELS

3.1 Introduction

By using previously obtained microarray data and localization data, treated RT2 cells will be stained and imaged for a host of select proteins. By imaging these cells at different time points, we will know the levels of proteins during different points in the action of Imipramine Blue. We will also be able to corroborate the microarray data and understand *when* Imipramine Blue begins to act on certain proteins. This data will help us narrow down our list of proteins to test for Imipramine Blue binding.

3.2 Methods

3.2.1 *in vitro* Immunocytochemistry

Cells were seeded in three 96 well glass-bottom plates (200 µl/well) at 50,000 cells/ml and incubated at 37°C in a 5% CO2 atmosphere. The cells were then treated with Imipramine Blue (5 mg/ml) for 0 hours, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours. After treatment, Imipramine Blue was removed and cells were washed with 1X PBS. Cells were then fixed using 4% paraformaldehyde and stained using the following primary antibodies: α-actin (1:100), scinderin (1:5 ug/ml), NFκB (1:100) , nox4 (1:100) (AbCam), profilin (1:100), rho-gdi (1:50) (Santa Cruz Biotechnology, Inc.), and acetylated microtubulin (1:200) (Invitrogen). After 24 hours, cells were stained with the following secondary antibodies: AlexaFluor 488 goat anti-rabbit, and AlexaFluor 488 goat anti-mouse (Invitrogen). The cells were then stained with

1X DAPI and phalloidin (1:200) obtained from Invitrogen. Cells were imaged using a Zeiss LSM 510 NLO with META MPE.

3.3 Results

3.3.1 Protein levels in RT2 and U87 glioma become variable in the presence of IB

After treating RT2, U87, and astrocyte cell lines with IB, the cells were fixed at different time points and stained with FITC for a host of proteins. Our observations through imaging clearly showed that the brightness of certain proteins would change, showing upregulation or downregulation. Over time, α-actin, profilin, NFκB, acetylated microtubulin, and Nox4 became less bright. Scinderin and rho-gdi, however, became brighter with time. We were not able to observe the first protein to change brightness.

3.3.2 IB does not alter actin formation in astrocytes

From observing the cell structure of IB treated cells in the assay, we could see that RT2 and U87 glioma became rounded and lost their invadopodia with time; astrocytes however were not affected and kept their shape after treatment. Staining for α-actin showed that the cytoskeleton of the glioma changed dramatically (Figure 5A). In addition, we went on to quantify the cell roundness in each treatment using Image J. We found that cell roundness in RT2 and U87 increased over time, but roundness in astrocytes did not increase at all (Figure 5B).

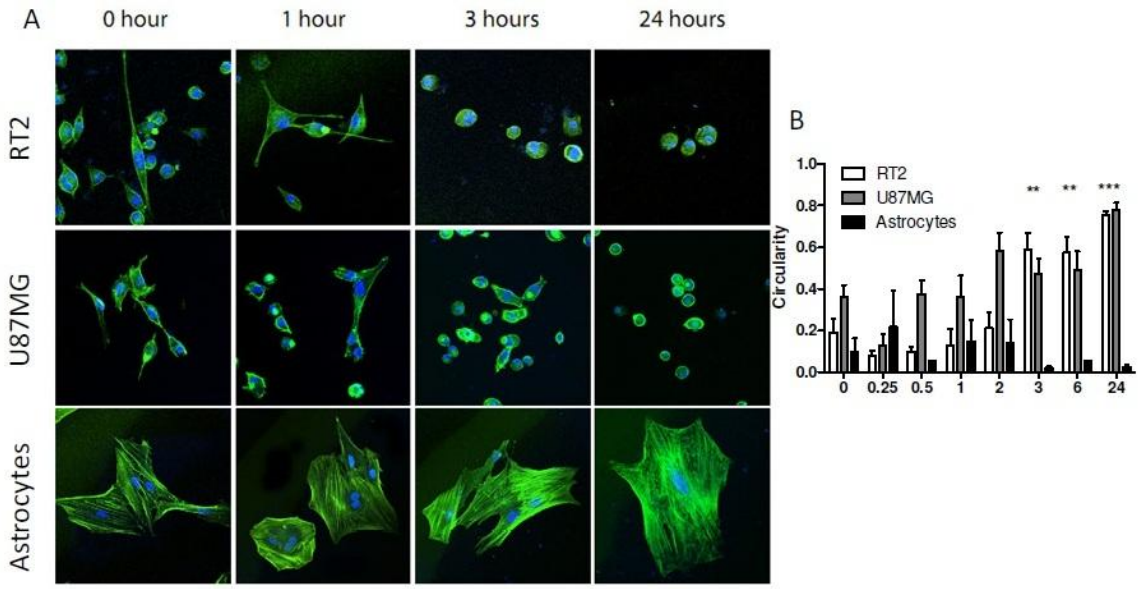

Figure 5: IB does not affect actin structures in astrocytes. (A) RT2, U87, and astrocytes were treated with IB and fixed at different time points. Cells were then imaged for F-Actin (green) and the nucleus (blue). RT2 and U87 show rounding after only 3 hours in response to IB treatment. Astrocyte cytoskeletons are unaffected, showing IB does not harm healthy cells. (B) Cell roundness was quantified from various images in the experiment. RT2 and U87 show increasing roundness with time, whereas astrocytes show very low roundness at all time points.

3.4 Discussion

The result of the experiment showed that IB changes protein levels *in vitro*, thereby upregulating or downregulating them (Figure 5). All of the proteins were in the same pathway, leading from RhoGDI to actin. However, it is not clear which protein's levels began to change. We also saw that the actin structures in the astrocytes were not affected by IB, meaning that IB has no affect on healthy cells or proteins. Our proteins of interest are likely mutated by the cancer, but we can still stain for them with normal antibodies. This experiment effectively confirmed that IB is cancer-selective and safe on healthy cells.

The data from this experiment also corroborated the microarray data obtained previously. The changes in upstream protein regulators cause changes in actin structures, making the ball like shape that is indicative of noninvasive cells.

CHAPTER 4

IMIPRAMINE BLUE PROTEIN INTERACTIONS

4.1 Introduction

Ultimately, we would like to find the specific protein that Imipramine Blue binds to so that we can fully understand it's function. This way, we can be assured that it is safe to use in animal and human trials in the long term. By using Desipramine Blue and conjugating it to Dynabeads®, which are magnetic, we can treat cells and hopefully bind the protein of interest. Then, by using the Western Blot technique, we will be able to establish the protein which is responsible for binding to Imipramine Blue, and thus halting invasion of the cancer cell. Previous data form collaborators indicates that Nox4 is likely the primary target for Imipramine Blue; because of this, we will stain the Western Blot for Nox4 in our experiments.

4.2 Methods

4.2.1 Actin polymerization and depolymerization assays

The effect of Imipramine Blue on actin polymerization and depolymerization was tested using the Actin Polymerization Biochem Kit (Cytoskeleton, Inc.) using the protocol provided with the kit. Cytochalasin-D (Invitrogen), a known inhibitor of actin polymerization was used as a negative control in the actin polymerization assay; phalloidin (Invitrogen), which inhibits actin depolymerization, was used as a negative control during the actin depolymerization assay.

4.2.2 Magnetic Bead Binding Assay

Dynabeads (Dynal Biotech, Inc.) were conjugated to DB using the protocol provided. Control beads were also made by activating according to the protocol and

withholding DB. Protein extracted from RT2 cells was treated with both the conjugated and unconjugated beads. The sorted protein from the conjugated beads, the supernatant from the conjugated beads, the protein from the unconjugated beads, the supernatant from the unconjugated beads, and the unsorted protein were all tested using a Western Blot. Based on recent data from a collaborating lab, the blot was stained with Nox4 primary antibody (AbCam) and stained with AlexaFluor488 goat-anti rabbit secondary antibody (Invitrogen).

4.3 Results

4.3.1 IB does not affect actin polymerization outside of cells

Although we saw that IB is cancer selective and does not affect the actin structures in healthy cells, we wanted to confirm that IB did not interact directly with the actin protein. Using the actin polymerization assay, we saw that actin polymerization outside of the cell was not affected by the presence of IB; the cytochalasin D control treatment showed a drastic decrease in actin polymerization. Conversely, we found that IB did not halt actin depolymerization in the actin depolymerization assay; phalloidin, which halts actin depolymerization showed a strong decrease in actin depolymerization (Figure 6).

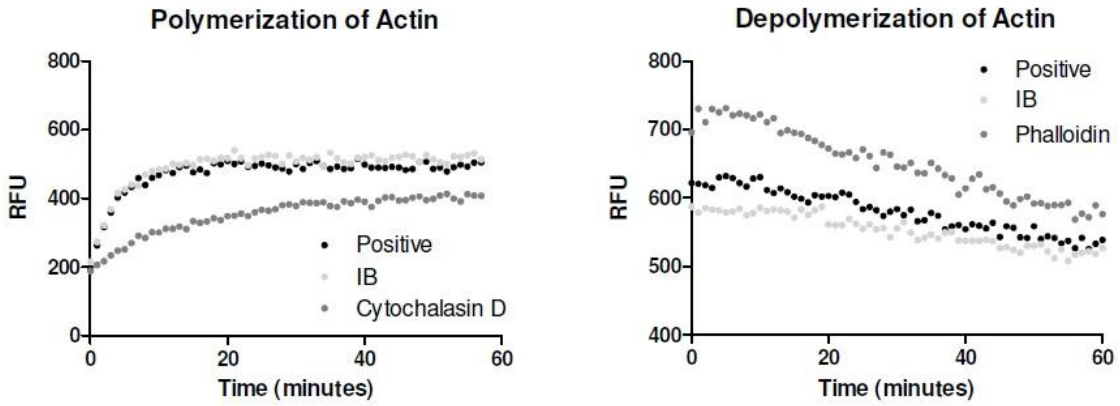

Figure 6: IB does not directly bind to actin. Actin polymerization and depolymerization assays were performed using IB, cytochalasin D, and phalloidin. The actin polymerization assay showed that IB had no effect on actin polymerization outside the cell; cytochalasin D, which halts actin polymerization showed slowed activity. IB also showed no effects on actin depolymerization; phalloidin however showed slowed depolymerization. Therefore, IB does not directly bind to actin.

4.3.2 Western blotting shows IB binds a 30 kDa protein

RT2 cells were first incubated with the DB-Dynabead conjugate to ensure that the cells underwent similar transformations to IB treatment. We observed that images taken after three hours showed similar actin morphology to IB treated cells; this morphology included cell rounding and loss of invadopodia. After performing the magnetic bead binding assay on RT2 glioma, a Western Blot was performed using the following treatments: beads alone, protein from unconjugated beads, protein before incubation with beads, the supernatant from pulled protein, and the pulled down protein itself. The blot was stained for Nox4 and showed no bands in any treatment except for the pulled protein at 30 kDa (Figure 7). The molecular weight of Nox4 is actually 70 kDa, indicating that the protein in question must be a variant of Nox4. Although Nox4 is present in RT2 and should have been stained in the protein before incubation treatment, we believe that the concentration of protein was greater after treatment with the beads, allowing for a stronger band.

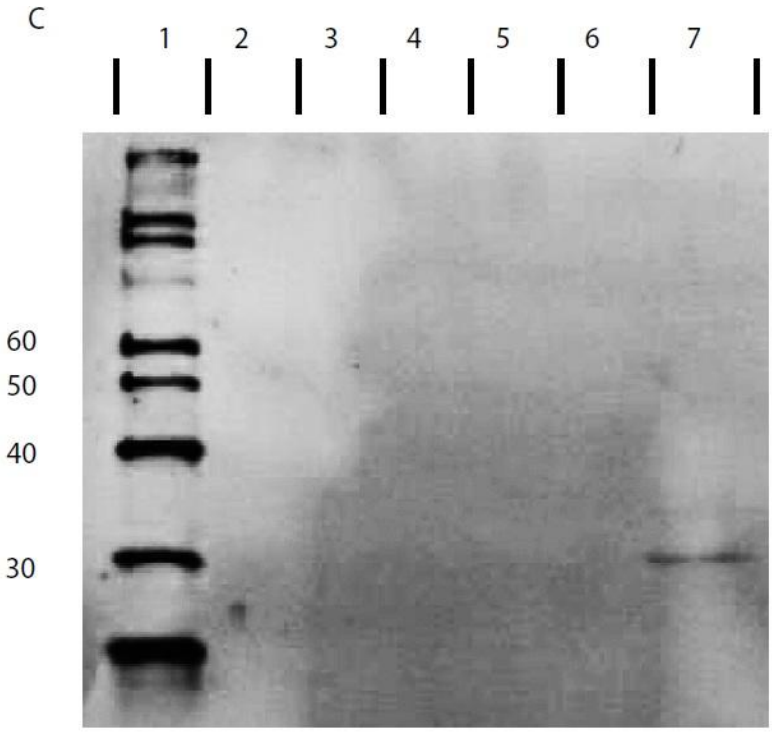

Figure 7: IB binds to Nox4 splice variant. Desipramine Blue, an analog of IB, was conjugated to Dynabeads. The protein was then isolated using the conjugate and identified using a Western Blot. The results showed a band at 35 kDA, implicating Nox4D as the protein.

4.4 Discussion

The first experiment verified that IB does not affect actin polymerization or depolymerization outside of the cell. Basically, the results mean that IB does not directly bind to or interact with actin when changing actin structures. This is clear because there are no upstream proteins in the actin polymerization assay. Therefore, we showed with no uncertainty that IB binds to an upstream protein.

The next experiment was done to find the specific binding protein, using the bead binding protein. We eventually decided to stain the Western Blot for Nox4 based on data from the Knaus lab at UCD showing that IB inhibits Nox4 activity. It was not clear if it was acting as a direct inhibitor, and we decided to find the answer using our Western Blot. The blot showed a band in the sorted protein column at 30 kDa; this result is odd

considering Nox4 weighs 75 kDa. Research by Goyal (2009) shows that Nox4 can be found as certain splice variants in cells. One of these, Nox4D, is 30 kDa, localizes to the nucleus, and has the same activity as Nox4. We believe that the protein from the Western Blot is Nox4D. This makes sense because Nox4D is a mutated protein, and may be the primary Nox protein acting in glioblastoma.

CHAPTER 5

DISCUSSION

1.1 Overall Discussion

The project overall showed that IB binds to and effectively inhibits Nox4D activity in RT2 glioma cells. This splice variant is located in the nucleus, which was confirmed by Specific Aim 1. Inhibition of Nox4D will cause the changes that we saw in the experiment in Specific Aim 2. If Nox4D is inhibited, it will cause the amount of ROS in the cell to be decreased, also causing inactivation of NFκB. Without NFκB, the transcription of actin mRNA into G-Actin, and thus polymerization of G-Actin into F-Actin is lowered (Figure 9). Knowing this specific mechanism of action can help us to be sure that IB is safe for use. Considering that its primary binding protein is mutated, it can be invaluable as a cancer therapeutic, especially when compared to therapeutics that are more harmful to the body than the cancer itself.

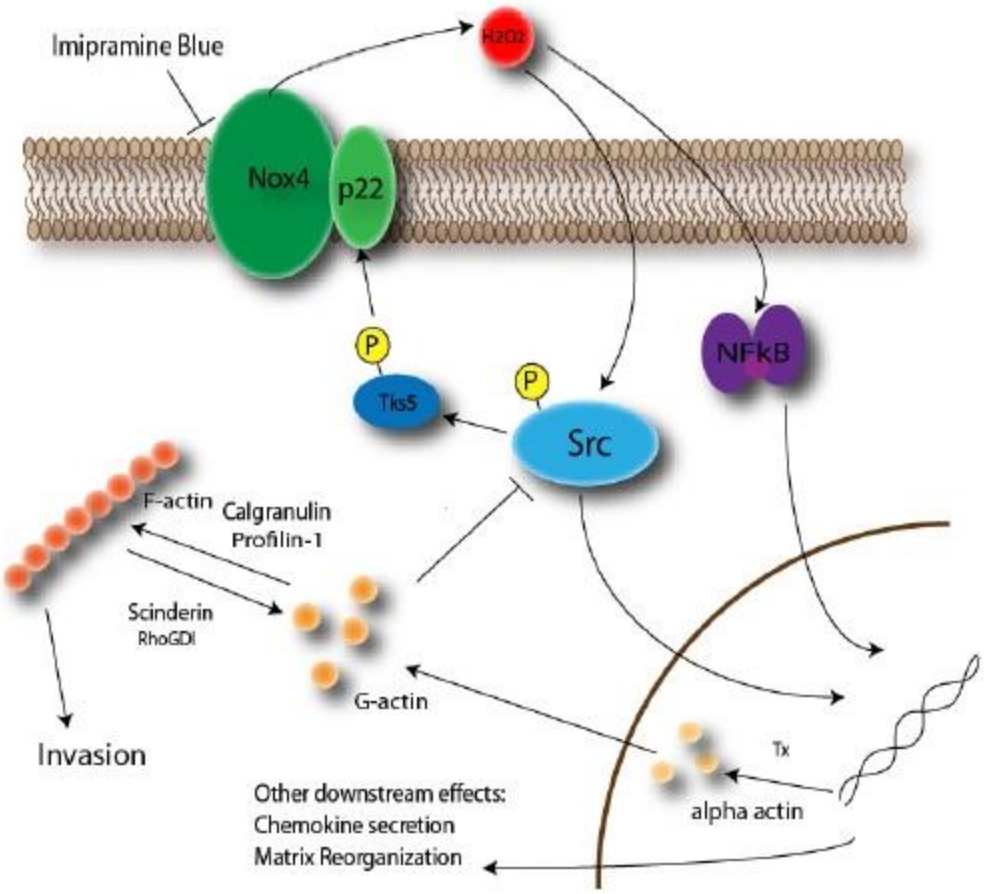

Figure 8: Mechanism of action of IB. The data shows that IB halts the activity of Nox4. This binding in turn leads to downstream effects including lowered signaling from second messengers and lowered actin formation. In all, this leads to inhibition of invasion. Adapted from Munson 2012.

1.2 Future Directions

Now that the mechanism of action of IB has been confirmed, it can hopefully be considered for use as a widespread cancer drug. In glioblastoma specifically, IB should begin to be tested in bigger animals; after that, it can be moved to human trials.

IB should also be tested in other cancer types and evaluated for its effectiveness in halting invasion in those types. If does halt invasion, the cancer should be tested for the presence of the Nox4D variant. However, drugs can have multiple targets, so it is

possible that IB can be binding to another protein. If IB can be used in multiple cancers, it would have extremely strong promise as a cancer therapeutic.

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VITA

MONA MELIHA AHMAD

AHMAD was born in Kitchener-Waterloo, Canada. She moved to Roswell, GA at a young age and went to Roswell High School and is currently pursuing her Bachelor's Degree in Biology at the Georgia Institute of Technology. She will be attending New York Medical College next year to earn her medical degree. While she is not studying, Mona enjoys painting and reading.